Non-Invasive Detection of Helicobacter pylori Infection

Field of the Invention

The present invention is directed to a kit and method for isolating amplifiable nucleic acid sequences from solid stool samples. More particularly, the invention relates to a non-invasive procedure for detecting pathogenic organisms by screening stool samples for the presence of the pathogenic organism's nucleic acid sequences.

Background of the Invention

In the United States alone, twenty-five million persons suffer from peptic ulcers, four million of them chronically. Approximately one million victims are hospitalized annually. *Helicobacter pylori*, a bacterium that was first isolated by Warren and Marshall in 1983, has been shown to play an important role in gastritis, peptic ulcer disease, and gastric malignancies.

H. pylori infections can be effectively treated with antimicrobial therapy once the proper diagnosis has been made. However, since *H. pylori* is found in the upper gastrointestinal tract of humans diagnosis is invasive and relatively expensive when endoscopy and gastric biopsy are employed. Accordingly, there is a need for a reliable non-invasive method for diagnosing *H. pylori* infections in humans.

Several diagnostic tests are available for determining the presence of *H. pylori* infection, however all existing tests suffer from one or more drawbacks. As noted above endoscopy followed by biopsy is one method to detect and to some extent localize Helicobacter, but this is a serious invasive procedure. Serological detection of antibodies against *H. pylori* is routinely employed to detect infections. Unfortunately, antibodies generated against *H. pylori* continue to persist even after eradication of the organism and thus are not a reliable indicator of an individual in need of treatment. A breath test, involving the consumption of radioactive carbon, in the form of ^{13}C and ^{14}C labeled urea with a beverage has been approved, but this test has the drawback of requiring the administration of radioisotopes to patients.

Molecular techniques such as PCR and Southern blot hybridization offer the capability to sensitively and accurately determine both the presence of

infection and the genotype of the bacteria. These techniques have been used to successfully detect *H. pylori* DNA in gastric tissue by amplifying genes such as the adhesin gene (Evans et al. American Journal of Gastroenterology 1995, 90(8), p.1282-1288), the urease gene (Clayton et al., Journal of Clinical Microbiology, 1992 30(1) p. 192-200) and the 16S ribosomal RNA (rRNA) gene (Ho et al., Journal of Clinical Microbiology, 1991 29, p. 2543-2549). The l6S rRNA gene of *H. pylori* is a highly specific target for amplification and has been used previously to help reclassify the organism. Weiss et. al. demonstrated the specificity of unique *H. pylori* 16S rRNA gene primers to identify the organism in paraffm-embedded gastric biopsy specimens (Journal of Clinical Microbiology, 1994 32(7) p. 1663-1668).

Stool analysis provides a noninvasive means of detecting *H. pylori*, and culturing stool samples has allowed detection of the urease gene by PCR (Kelly et al., Gastroenterology, 1994, 107, p. 1671-1674). However, the sensitivity of this assay was low and the ability to routinely culture stools for this purpose unproven. Direct PCR analysis of nucleic acids recovered from stool samples has been used successfully to diagnose several infections including Rotovirus (Ushijima et al., Journal of Medical Virology, 1992 38 p. 292-297), Microsporidia (Da Sila et al., Arch Pathol Lab Med 1997, 121 P. 874-879), *Vibrio cholerae* (Albert et al., Journal of Clinical Microbiology, 1997 35(6) p. 1633-1635), verotoxin producing *Escherichia coli* (Ramotar et al., Journal of Clinical Microbiology, 1995 33(3) p. 519-524, and Salmonella (Chiu et al., Journal of Clinical Microbiology, 1996 34(10) p. 2619-2622). However, in each of those instances the disease state causes diarrhea. The method used to isolate and amplify DNA from the stool samples of individuals suffering from Microsporidia, Rotovirus, *Vibrio cholerae* and verotoxin producing *Escherichia coli* infections does not provide a consistent and reliable method for obtaining amplifiable DNA from normal stool samples. Similarly, although PCR analysis of stool has detected mutations of K-ras from tumor cells shed from colonic neoplasms (Sidransky et al., Science, 1992, 256, p. 102-105) that method also failed to reliably isolate amplifiable DNA from normal stool samples. Simply stated, there has not been a reported case of diagnosing a bacterial infection based on PCR analysis of a normal stool sample.

Previous reports of direct PCR analysis of stool samples for *H. pylori*

have shown low sensitivity (see Zwet et al., J. Clin. Microbiol. (1994), 32:1346-1348) and poor reliability. Zwet et al. concluded that "[t]herefore, we believe that the use of fecal samples for the detection of *H. pylori* in patient is precarious." The difficulty in direct PCR amplification of stool samples is generally thought to be related to enzyme inhibitors present in normal stool samples.

The present invention provides a method and kit for isolating nucleic acids from normal solid stool samples and in a form that is capable of being amplified by polymerases used in PCR reactions. The non-invasive method of the present invention is used to detect *H. pylori* in the feces as a diagnostic test for the disease as well as a means to monitor the effectiveness of therapy during treatment. Furthermore, certain *H. pylori* genotypes, such as strains possessing the cagA gene or vacA gene, may be more virulent than others. PCR analysis of nucleic acids recovered from stool samples also allows for the non-invasive identification of the specific strain of *H. pylori* bacteria. Thus the method is capable of determining the virulence of the infection based on the presence or absence of specific *H. pylori* nucleic acid sequences. Accordingly the present invention is an improvement over the currently available diagnostic tools used to identify *H. pylori* infections.

Summary of the Invention

The present invention is directed to a method for isolating amplifiable nucleic acids from a solid stool sample. Isolation of amplifiable nucleic acids from stool samples allows for the characterization of those nucleic acid sequences by PCR analysis to identify the presence of pathogenic organisms. Thus the presently described method can be used as a diagnostic tool for determining the health of an individual. In accordance with one embodiment, the method is used to screening for an *H. pylori* infection in a patient. The method comprises the steps of obtaining a stool sample from the patient, removing lipid and protein contaminants and inhibitors of DNA polymerases, isolating the DNA and subjecting the DNA to PCR analysis using PCR primers specific for *H. pylori* nucleic acid sequences.

Detailed Description of the Invention

Definitions

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below.

As used herein, "nucleic acid," "DNA," and similar terms also include nucleic acid analogs, i.e. analogs having other than a phosphodiester backbone. For example, the so-called "peptide nucleic acids," which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention.

As used herein, the term "probe" refers to an oligonucleotide (i.e., a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, which is capable of hybridizing to another oligonucleotide of interest.

The term "label" as used herein refers to any atom or molecule which can be used to provide a detectable (preferably quantifiable) "signal", and which can be attached to a nucleic acid or protein. Labels may provide "signals" detectable by fluorescence, radioactivity, colorimetry, gravimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like.

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (i.e., a sequence of nucleotides) related by the base-pairing rules. For example, for the sequence "A-G-T," is complementary to the sequence "T-C-A." Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods which depend upon binding between nucleic acids.

The term "homology" refers to a degree of complementarity. There may be partial homology or complete homology (i.e., identity). A partially complementary sequence is one that at least partially inhibits a completely complementary sequence from hybridizing to a target nucleic acid is referred to using

the functional term "substantially homologous."

As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (i.e., the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementary between the nucleic acids, stringency of the conditions involved, the Tm of the formed hybrid, and the G:C ratio within the nucleic acids.

As used herein, the term "Tm" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the Tm of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the Tm value may be calculated by the equation: $Tm = 81.5 + 0.41(\% G + C)$, when a nucleic acid is in aqueous solution at 1M NaCl (see e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985).

As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic solvents, under which nucleic acid hybridizations are conducted. With "high stringency" conditions, nucleic acid base pairing will occur only between nucleic acid fragments that have a high frequency of complementary base sequences.

"Amplification" is a special case of nucleic acid replication wherein the concentration of a nucleic acid sequence is increased. One method of amplifying DNA sequences utilizes the polymerase chain reaction (PCR).

As used herein, the term "amplifiable nucleic acid" is used in reference to nucleic acids which may be amplified by any amplification method.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (i.e., in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH).

As used herein, the term "polymerase chain reaction" ("PCR") refers to

the method of K. B. Mullis U.S. Pat. Nos. 4,683,195 and 4,683,202, hereby incorporated by reference, which describe a method for selectively increasing the concentration (i.e. "amplification") of a target nucleic acid sequence present in a mixture of nucleic acid sequences without cloning the target nucleic acid sequence.

As used herein, "extract" and similar terms relates to the treatment of a composition (a solid or liquid) with a solvent so as to separate and remove a soluble substance from the composition.

As used herein, "rinsing" is intended to mean cleansing by flushing with a liquid.

As used herein, "solubilize" refers to the dissolution of at least a portion of a solid or semisolid substance in a solvent.

A chaotropic agent as used herein is an agent that is capable of disrupting the membranes or other structural components of living organisms and includes but is not limited to urea, enzymes such as lysozyme, alkali solutions, chelators such as EDTA and EGTA and detergents such as SDS, Tween, TritonX and Sarkosyl.

As used herein, "effective amount" means an amount sufficient to produce a selected effect. For example, an effective amount of an antibiotic for treating an *H. pylori* infection is an amount of antibiotic sufficient to decrease the *H. pylori* population to a level that alleviates the symptoms associated with such an infection.

Disclosure of the Invention

The present invention is directed to a method of recovering nucleic acid sequences from fecal samples in such a manner that the recovered nucleic acid sequences can be replicated using standard techniques. Advantageously, the present invention allows the recovery of amplifiable nucleic acids from normal stool samples without the use of expensive chromatographic techniques. In one preferred embodiment the recovered nucleic acid sequences are bacterial DNA that are amplified by PCR.

To recover amplifiable nucleic acid sequences from stool samples, the stool sample, or portion thereof, must first be dispersed in a sample diluent. Typically

the stool sample is suspended in an aqueous solvent using standard techniques. In a preferred embodiment the sample is first suspended in a lipid solubilizer solution. For example, the solvent used to resuspend the stool sample may comprise an alcohol/chloroform mixture, wherein the ratio of alcohol:chloroform ranges from about 70:30 to about 95:5. The alcohol can be selected from any lower alkyl (C_1-C_4) alcohol, and more preferably methanol or ethanol. After the stool sample has been resuspended the mixture is centrifuged at a speed that pellets the cellular material and leaves the lipids remaining in the supernatant. For example, the resuspended mixture can be centrifuged at about 2000 to about 2200g to separate the mixture into a solid mass and a liquid supernatant. The supernatant is then discarded. The pellet can be subjected to repeated rounds of resuspension with the lipid solubilizer solution and centrifugation at about 2000 to about 2200g to ensure removal of substantially all of the lipids from the stool sample. Finally the pellet is optionally rinsed with a solvent (such as acetone).

After the lipid solubilization and removal step, the pelleted mass is resuspended in a cell lysis solution, wherein the cell lysis solution comprises a chaotropic agent. The mixture is incubated for a time sufficient to lyse any cellular material present in the original stool sample. The incubation time can be varies and depends on the chaotropic agent used, the concentration of the chaotropic agent(s) and the temperature of the mixture. Typically, the cell lysis solution will comprise an ionic or nonionic detergent, and a buffering agent and will be incubated at about 37°C to about 60°C for about 30 to about 90 minutes. The detergent can be selected from the group consisting of SDS, NP40, Tween, Triton-X, Guanidium or any other commercially available detergent. Any standard buffering agent can be used, with Tris being the preferred buffering agent. In accordance with one embodiment the lysis solution comprises an 8M solution of urea containing a detergent and a buffering agent, and in one preferred embodiment the lysis solution comprises an 8M solution of urea containing SDS and Tris.

Once the mixture has been incubated for a sufficient length of time to lyse a majority of the cellular material, the mixture is heated at about 90°C to about 100°C, and more preferably boiled, for about 5 to about 15 minutes to produce a lysed cell solution containing a precipitate. The precipitate is then separated from the lysed

cell solution and removed. Typically, the precipitate is separated from the lysed cell solution by centrifuging the sample at about 400 to about 500g and recovering the resulting supernatant.

The lysed cell solution is then treated with enzymes selected from the group consisting of RNases and proteases. In accordance with one embodiment the lysed cell solution is contacted with a solution comprising protease K, and more preferably the enzymatic solution comprises protease K and an RNase. In one embodiment, the lysed cell solution is first extracted with an organic solvent before being contacted with the enzymatic solution. For example, the lysed cell solution can be organically extracted, and the nucleic acids precipitated with alcohol using standard techniques. The resulting pellet is then resuspended in a solution comprising NaCl and CTAB and incubated at about 65°C for about 30 to about 90 minutes. The solution is then contacted with enzymes selected from the group consisting of RNases and proteases.

After the mixture has be enzymatically treated, the mixture is extracted with an organic solvent to produce an aqueous and organic phase. In accordance with one embodiment the organic solvent used is phenol, and more preferably a phenol/chloroform mixture. The nucleic acids are then recovered from the aqueous phase using standard techniques. In one preferred embodiment the nucleic acids are recovered by alcohol or Lithium chloride precipitation of the nucleic acids. Other means of purifying DNA can be used in accordance with the present invention, including the use of affinity columns or matrices (such as glass beads), anion exchange, salt binding and other techniques known to the skilled practitioner. In accordance with one embodiment the recovered nucleic acid is DNA and more particularly *H. pylori* DNA.

The method of isolating amplifiable nucleic acids can be used as a screen for detecting an *H. pylori* infection in a patient. The method comprises the steps of obtaining a stool sample from the patient, recovering amplifiable DNA from the stool sample and conducting PCR using *H. pylori* specific primers. The amplified DNA is then analyzed using standard techniques to determine if *H. pylori* DNA is present. In accordance with one embodiment the PCR reaction is conducted in the present of radioactive nucleotides and the PCR reaction products are electrophoresed

on a gel to determine if the target *H. pylori* sequence was present in the original stool sample. Alternatively, the PCR reaction can be conducted with unlabeled nucleotides and the PCR reaction products are electrophoresed on a gel and blotted and probed using standard Southern blot analysis.

In accordance with one embodiment, a method for detecting an *H. pylori* infection in a patient comprises the steps of obtaining a stool sample from the patient, solubilizing said stool sample in a solvent comprising alcohol and chloroform, and centrifuging the solubilized stool sample at about 2000 to about 2200g to separate the mixture into a solid mass and a liquid supernatant. The supernatant is then discarded and the pellet is rinsed with acetone. The solid mass is then resuspended in a solvent comprising a chaotropic agent to produce a lysed cell solution. The lysed cell solution is then heated at about 90°C to about 100°C to produce a heat treated lysed cell solution containing a precipitate. The precipitate is then removed from the heat treated lysed cell solution and the heat treated lysed cell solution is incubated with an enzyme selected from the group consisting of RNases and a proteases to produce an enzyme treated solution. The enzyme treated solution is then extracted with an organic solvent to produce an aqueous and organic phase and the nucleic acid is isolated from the aqueous phase using standard techniques.

The isolated DNA is then amplified by PCR amplification using primers that are specific for *H. pylori* nucleic acid sequences and the PCR products are analyzed for amplified *H. pylori* nucleic acid sequences. In one embodiment the *H. pylori* specific primers are complementary to nucleic acid sequences that relate to the virulence of the *H. pylori* strain. For example, the oligonucleotide probes can be complementary to nucleic acid sequences encoding the *vac A* or *cag* A *H. pylori* genes.

The present invention also encompasses a kit that contains the necessary reagents for analyzing stool samples for the presence of *H. pylori* nucleic acids. In accordance with one embodiment the kit comprises a chaotropic agent, an enzyme and an oligonucleotide primer specific for *H. pylori* nucleic acid sequences. The chaotropic agent of the kit will typically include a detergent and/or urea, and in one embodiment the chaotropic agent is a solution comprising SDS and 8M urea. The enzyme will typically be in the form of a solution containing a protease, and

optionally an RNase as well. In one preferred embodiment the protease is protease K.

Example 1

Isolation and Analysis of DNA Recovered from Stool Samples

To investigate the ability of using PCR analysis of stool samples as a diagnostic tool, the followin experiments were conducted. Stool, gastric biopsies, and serum were collected from 22 subjects. DNA from stool was extracted, amplified, and hybridized with primers specific for the 16S rRNA gene of *H. pylori*. DNA from gastric biopsy specimens was analyzed similarly for comparison. Universal primers were used to confirm successful DNA extraction of samples. Histologic, serologic, and DNA analysis were scored in a blinded fashion.

Materials and Methods

Patients undergoing upper endoscopy were recruited and Esophagogastroduodenoscopy was performed on all subjects using endoscopes that had been sterilized by a Steris (Mentor, Ohio) machine. Autoclaved biopsy forceps were used to obtain gastric biopsies from the antrum for rapid urease testing (CLOtest). Gastric tissue was also obtained from the antrum, the incisura, and the body of the stomach for histologic examination and DNA analysis. Stool specimens were collected within two weeks of the time of endoscopy in sterile containers for freezing at -80° C until analysis. Blood from all patients was collected and the serum stored at -20°C until the EIA test of IgG antibody response to *H. pylori* was performed using an FDA approved, commercially available kit (HM-CAP EIA kit, Enteric Products, Stonybrook NY).

Zinc formalin-fixed paraffin-embedded biopsy specimens were stained with H&E and Giemsa. A single pathologist (HF) scored all gastric biopsy specimens without knowledge of the results of the other tests. The number of *H. pylori* organisms was semi-quantitatively scored as 0, 1 (few; organisms present but difficult to find and rare in 400x fields), 2 (moderate; organisms readily identified upon microscopic examination and present in most 400x fields), and 3 (numerous; organisms present in virtually all 400x fields).

DNA extraction:

One gram of stool from each patient was dissolved in 100% ethanol and chloroform, then centrifuged at 2135 g, and rinsed with acetone. The sample was then mixed with 8 M urea containing SDS, Tris, Chelex (BioRad, Hercules, Ca), and PVPP for subsequent incubation at 60°C. The samples were then boiled and centrifuged at 469 g. The supernatant was organically extracted, precipitated with alcohol, and re-dissolved with sodium chloride and CTAB for incubation at 65°C. Organic extraction and alcohol precipitation was performed for subsequent RNase and proteinase K incubation at 58°C for 2 hours. Another round of organic extraction and alcohol precipitation was preformed with reconstitution in a Tris buffer.

Gastric tissue DNA extraction from paraffin-embedded specimens was performed using xylene and SDS/proteinase K on two 5 micron thick sections as previously described Moskoluk et al., American Journal of Pathology, 1997 150(5) p. 1547-1549. Cultured *H. pylori* DNA extraction was conducted on an *H. pylori* isolate from a human subject confirmed to have this infection. *H. pylori* cultured on horse blood agar plates was scraped into one ml of phosphate buffered saline. An aliquot of this suspension was then incubated overnight with proteinase K prior to organic extraction and alcohol precipitation. The optical density was measured in the re-dissolved pellet for quantitation and subsequent serial dilutions. Concentrations as low as 1 femptogram of DNA per uL were generated. A single bacterial genome was considered equivalent to 1.6 femptograms ofDNA.

PCR Amplification

Universal Primers. PCR amplification with non-specific, "universal" primers was performed in 25 uL reaction volumes containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 200 uM dNTPs, 10 pmoles of each primer, 2.5 units of Taq polymerase (Perkin-Elmer Cetus, Norwalk, Connecticut), and 1.5 uL of DNA template as prepared above. The universal primers for stool analysis consisted of two non-specific 16S rRNA oligonucleotides designated USA-l (AGAATGCCACGGTGAATACG; SEQ ID NO: 1) and USA-2 (CCTACGGTTACCTTGTTACG; SEQ ID NO: 2). Forty thermocycles were performed with each cycle consisting of a 30 second denaturation step at 95°C, a 60

second annealing step at 50°C and a 60 second extension step at 70°C. The universal primers for gastric tissue DNA analysis consisted of two oligonucleotides designed to amplify exon 7 of the *Smad4* gene. The forward primer (TGAAAGTTTAGCATTAGACAAC; SEQ ID NO: 3) and a reverse primer (TGTACTCATCTCAGAAGTGAC; SEQ ID NO: 4) were used in a similar PCR reaction as above with an annealing temperature of 50°C. These primers have previously proven to successfully amplify this exon for subsequent sequencing (Powell et al., Cancer Research, 1997, 57, p. 4221-4224).

H. pylori specific primers:

PCR amplification with *H. pylori* specific primers was performed similarly to the universal primer amplifications in 25 uL reaction volumes with the same buffer, dNTP, primer, and Taq polymerase concentrations. The thermocycle conditions were similar with an annealing temperature of 60°C. The primers consisted of two specific 16S rRNA oligonucleotides designated HPF (GCGACCTGCTGGAACATTAC; SEQ ID NO: 5) and HPR (CGTTAGCTGCATTACTGGAGA; SEQ ID NO: 6) generating a 138 bp product. In all PCR amplifications, the final cycle was a 5 minute extension step at 70°C to allow full product extension. Each experiment included a negative control template consisting of water and a positive control consisting of 100 fg of cultured *H. pylori* DNA.

Southern Blot Hybridization

Half of the PCR products were electrophoresed on 2% agarose gels and transferred to nylon membranes (Biorad, Hercules, CA) and hybridized with an end-labeled probe (1×10^6 cpm/ml) in a standard Southern blot fashion. The probe was a 16 base pair oligonucleotide (CGCTGATTGCGCGAAA; SEQ ID NO: 7) designed specifically for a region within the 16S rRNA gene of *H. pylori* as previously described (Weiss et. al., Journal of Clinical Microbiology, 1994 32(7) p. 1663-1668) and was labeled in a [^{32}P]-dATP T4 kinase reaction according to manufacturer's instructions (New England Biolab, Beverly, MA). Autoradiographs generated clear signals after an overnight exposure at room temperature. The autoradiographs were

scored by two independent observers, each of whom was blinded to the results of the other tests, with a signal in the expected location recorded as either present or absent.

Results

Detection of *Helicobacter pylori* DNA

The sensitivity of the molecular assay was assessed by determining the threshold for *H. pylori* DNA detection. A clinical isolate of *H. pylori* was grown in culture, DNA was extracted, and quantitated. Dilutions of this cultured *H. pylori* DNA were PCR amplified with specific *H. pylori* 16S rRNA primers prior to subsequent hybridization. Specific *H. pylori* signals were found with amounts as low as 10 femptograms, which corresponds to less than seven genome equivalents of *H. pylori*.

H. pylori Status in Human Subjects

Twenty-two cases had specimens collected consisting of gastric biopsy tissue for CLO-test, histology, and PCR amplification; serum for ELISA, and stool for PCR amplification. Eleven of the 22 subjects were *H. pylori* positive by histologic and serologic analysis, while ten of these cases were positive by the CLO-test Endoscopic findings, medication use, and results of these conventional tests for *H. pylori* are summarized in Table I.

Table 1. Clinical features

Case Number	Acid Suppression Medication#	Endoscopic Findings	CLOtest	Serology	Histology (grade)*
1	none	normal	positive	positive	positive(1)
2	Ranitidine 150 bid	gastric	negative	negative	negative(0)
3	Omeprazole 20 bid	normal	negative	negative	negative(0)
4	none	duodenal dilation	negative	negative	negative(0)
5	Famotidine 20 qd	esophagitis	negative	negative	negative(0)
6	Nizatidine 150 bid	duodenitis	negative	negative	negative(0)
7	Nizatidine 150 qd	antritis	positive	positive	positive(2)
8	Ranitidine 150 bid	duodenitis	positive	positive	positive(1)
9	none	duodenitis	negative	negative	negative(0)
10	Ranitidine 150 bid	normal	positive	positive	positive(2)
11	Omeprazole 20 qd	Antritis, esophagitis	negative	negative	negative(0)
12	none	esophageal nodule	negative	negative	negative(0)
13	Nizatidine 150 bid	gastritis	negative	negative	negative(0)
14	Nizatidine 150 bid	duodenitis	positive	positive	positive(2)
15	none	Schatzki's ring, antritis	positive	positive	positive(3)
16	Nizatidine 150 bid	duodenal ulcer	negative	negative	negative(0)
17	Famotidine 20 qd	erosive antritis, duodenitis	negative	negative	negative(0)
18	none	erosive esophagitisitis	positive	positive	positive(2)
19	none	prepylotic ulcer	positive	positive	positive(2)
20	none	gastic ulcer	positive	positive	positive(2)
21	none	gastritis, duodenitis	positive	positive	positive(2)
22	Omeprazole 20 bid	esophagitis, antritis	positive	positive	positive(2)

Numbers represent milligram doses; qd=once daily; bid=twice daily.

* Semiquantitation of the number of *H. pylori* organisms: 0=none; 1=few; 2=moderate; 3=numerous

Gastric Biopsy DNA Analysis

Amplifiable DNA extracted from the gastric biopsy specimens was achieved from each of the 22 study subjects. A 224 bp PCR product from exon 7 of *Smad 4* was successfully generated and seen on ethidium bromide stained agarose gels for each case. These same DNA templates were amplified and hybridized with oligonucleotides specific for *H. pylori*'s 16S rRNA gene generating positive signals in 11 of the 22 patients. Independent PCR amplification and hybridization experiments confirmed these results. The autoradiographic signals were easily scored with 100% agreement by each of the two observers. Our DNA analysis results completely agreed with the histological and serological findings for these same subjects (see Table I).

The sensitivity of this molecular assay was further illustrated by gastric tissue testing of 24 additional cases. For two cases, the molecular assay detected a clear but weaker signal when gastritis was present histologically, but no demonstrable organisms found even on analysis of additional stained sections. Both of these cases were positive by serology but negative on CLO-tests, and both had been treated with antiacid secretory therapy prior to examination. Complete agreement on histology, serology and DNA analysis for the other 22 gastric biopsy cases was found.

Stool DNA Analysis:

Several methods of stool DNA extraction were tested from subjects with and without *H. pylori* infection as determined by conventional testing. The amount of DNA recovered varied depending on the protocol used. The presently described novel method of extraction was developed using lipid solubilizers, ionic and non-ionic detergents, chelators, and organic solvents, which routinely produced amplifiable DNA. One gram of stool consistently generated approximately 10 ug of DNA. Identifiable signals were repeatedly generated when 200 ng of DNA were used as a PCR template in the presently described assay. At least three independent experiments of stool DNA extraction, PCR amplification, and hybridization for 10 cases (five *H. pylori* positive and five *H. pylori* negative) consistently identified the presence or absence of *H. pylori* DNA, confirming the reproducibility and accuracy of the molecular assay.

To determine the sensitivity and specificity of the assay in identifying

H. pylori infection, frozen stool samples were collected from the 22 study subjects and extracted for DNA according to the presently described protocol. The samples were then analyzed for *H. pylori* DNA, and the results compared to the findings of conventional testing and gastric tissue DNA analysis. Extracted stool samples were first amplified with universal primers for the 16S rRNA gene to demonstrate the presence of amplifiable DNA. A clearly visible PCR product of expected size (148 bp) was generated in all samples as seen by ethidium bromide staining of agarose gels. Subsequent amplification and hybridization of these same stool DNA templates with primers specific for the *H. pylori* 16S rRNA gene resulted in positive signals in 8 subjects. Moreover, the twelve other subjects had unambiguous negative signals for *H. pylori* DNA. Independent PCR amplification and hybridization of all DNA samples confirmed these results. Compared with histology, serology, and gastric DNA analysis, the molecular stool DNA assay had a sensitivity of 73% and a specificity of 100%.

Discussion

Currently available tests for the diagnosis of *H. pylori* infection have relatively high sensitivities and specificities but each has its limitations in clinical application. Urease-based biopsy tests require endoscopy, and are not reliable in patients taking proton pump inhibitors. Histologic examination also follows endoscopy, is subject to sampling error, and its accuracy is dependent on the stain selected and on the pathologists' skill. Serology is inexpensive, but is not reliable in determining the presence of an active infection. Moreover, none of the currently available non-invasive tests provide information on the specific strain of *H. pylori* present.

The successful amplification and detection of *H. pylori* DNA directly from stool samples demonstrates that such a procedure can be useful for the diagnosis and management of patients with *H. pylori* infection. The surprising sensitivity of *H. pylori* DNA detection of 73% from uncultured stool specimens far exceeds previous reported results and results from the novel extraction method described in the present invention. The sensitivity of PCR amplification is well-known and is exhibited by our ability to easily detect DNA from less than seven *H. pylori* bacterial organisms. The

extreme sensitivity and specificity of the present molecular assay in analyzing gastric tissue biopsy samples has been demonstrated in both the study subjects as well as in an additional set of cases. In two of these additional cases in which infection was felt to be present serologically and histologically by the pattern of gastritis, *H. pylori* DNA was detectable, but organisms could not be identified microscopically. Notably, these two patients had prior anti-acid secretory therapy.

In addition, the data demonstrates that the presently described molecular assay is specific for *H. pylori* DNA in human stool and gastric specimens. The *H. pylori* 16S rRNA primers used were designed from those tested by Weiss et. al. (Journal of Clinical Microbiology, 1994 32(7) p. 1663-1668) who demonstrated their specificity in detecting *H. pylori* DNA in paraffin embedded gastric tissue. Furthermore, they found that these primers did not cross-react with many other common microorganisms such as *Escherichia coli*, various Camplyobacter species, and other Helicobacter species including *H. cinnaedi*, *H. fenelliae*, and *H. muselae*. Scholte et. al. (Diagnostic Molecular Pathology, 1997, 6(4) p. 238-243) have tested primers designed to amplify this similar unique region of 16S rRNA on 38 different bacteria, including 10 Helicobacter species and found 100% sensitivity and specificity.

The DNA analyses of human stool and gastric biopsy samples using these specific oligonucleotides correlated well with conventional tests for *H. pylori* infection. These results also suggest that sampling error is not a major obstacle in the detection of *H. pylori* DNA in stool samples. Small portions of spontaneously passed stool yielded consistent results on repeated analyses, and identified the majority of infections. The reason for the failure to identify *H. pylori* DNA in three stool samples from individuals known to have *H. pylori* organisms and DNA in their gastric tissue is uncertain. Sampling error certainly could have played a role in these three cases as could have degradation of DNA and organisms during intestinal transit.

The molecular detection of *H. pylori* DNA has the added benefit of being able to genotype the infecting strains, and provide useful information on the presence or absence of virulence factors for a particular infection. For instance, once virulent components of *H. pylori* are characterized (such as the vacA gene variants or cagA gene), PCR primers can be designed to specifically identify these important

variants. The presence of certain genotypes of vacA and cagA in infecting strains has been shown to be associated with a more dense inflammatory gastritis and peptic ulcer disease (Atherton et al., Gastroenterology, 1997, 112, p. 92-99).

These results demonstrate the feasibility of detecting *H. pylori* DNA non-invasively, and provide evidence for the frequent presence of *H. pylori* DNA in stools of infected patients.